Original article

Constraints upon cheating emergence in mutualistic networks: evidence in the mycorrhizal symbiosis

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Abstract

While multiple-partner mutualisms are widespread and essential in ecosystems, cheaters often exploit mutualistic interactions and threat their stability. The persistence of mutualisms relies on constraints upon cheating emergences: cheaters can be constrained by interaction mechanisms preventing cheating or phylogenetic conservatisms of interactions. By investigating the structures of interaction networks and the patterns of phylogenetic distributions of cheaters, we provide an integrative framework to evaluate the strength of constraints upon cheaters in the mycorrhizal mutualistic symbiosis, linking land plants to fungi, where non-photosynthetic plants (mycoheterotrophs) repeatedly evolved. Unlike mutualistic autotrophic plants, cheaters appeared narrowly specialized toward some closely-related specialist fungi. This shows that (i) cheaters are specifically isolated into modules by interaction constraints, and (ii) mycoheterotrophic lineages convergently interacted with 'cheating-susceptible' fungal partners, breaking any phylogenetic conservatism.

Beyond mycorrhizal systems, our approach highlights that cheating emergences in multiple-partners mutualisms are strongly limited by interaction constraints, ensuring the overall stability of mutualism.

Keywords: mutualistic networks, cheating, stability of mutualism, constraints upon cheating, nestedness, symbiosis, interactions, modularity, arbuscular mycorrhiza, mycoheterotrophy.

Introduction

Mutualistic interactions are ubiquitous in nature and largely contribute to the origin and maintenance of biodiversity (Bronstein 2015). In mutualism, benefits often come at a cost for partners; hence some species evolved an adaptive uncooperative strategy by retrieving benefits from an interaction without paying the associated cost, namely *cheaters* (Sachs et al. 2010). Cheating strategies compromise the evolutionary stability of mutualisms (Ferriere *et al.* 2002), yet their emergence must be constrained by factors that guarantee the persistence of mutualism (Bronstein *et al.* 2003; Frederickson 2013; Jones *et al.* 2015). For instance, species could favor the most rewarding partners (e.g. conditional investment; Roberts & Sherratt, 1998), stop interactions with non-beneficial partners (Pellmyr & Huth, 1994), or even sanction them (Kiers et al. 2003). Cheating emergence would thus be constrained through physiological or biochemical mechanisms at work during the interaction; this type of constraints is hereafter referred as *interaction constraints*. In addition, ultimate factors such as *phylogenetic constraints* could affect the likelihood of cheaters emergence, especially if species tend to retain an ancestral interaction niche (i.e. partners or some functional traits; Gómez *et al.* 2010). When partners are vertically transmitted, the absence of partner choice strongly impairs the rise of cheaters (Sachs et al. 2004).

Understanding the constraints that actually limit the emergence of cheaters in mutualisms is essential, but we lack a general framework for this purpose. Nonetheless, bipartite networks—can be used as frameworks to assess the relative effects of interaction constraints on cheaters emergence. Although bipartite networks have been extensively used to showcase the properties of mutualistic interactions (Bascompte et al. 2003, Rezende *et al.* 2007, Martos et al. 2012), there is still little insight on the

68 patterns of cheaters within mutualistic networks (e.g. their level of *specialization*,
69 *nestedness,* or *modularity)*, and the constraints shaping them (Fontaine et al. 2011).
70 Regarding specialization, Joffard et al. (2018) for instance showed that orchid
71 specialization toward pollinators was higher in deceptive cheaters than in cooperative
72 nectar-producing species. Considering the overall effect of cheaters on network
73 structure, Genini et al. (2010) showed that a network dominated by actual pollinators
74 was nested, whereas another network dominated by nectar thieves was more modular.
75 Mutualistic interactions are generally nested, i.e. specialists establish asymmetric
76 specialization with highly generalist partners (Bascompte & Jordano 2014). Conversely,
77 a high modular structure, where partners establish reciprocal specialization, is expected
78 in antagonistic interactions (Thebault & Fontaine 2010). Coupled with phylogenetic
79 trees, bipartite networks reflect the strengths of phylogenetic constraints. It determines
80 whether cheaters emerged a unique *versus* several times (i.e. whether cheating involves
81 some rare and constrained evolutionary innovations to change the functioning of the
82 interaction; Pellmyr *et al.* 1996), and whether 'cheating-susceptible' partners are
83 phylogenetically related or not (i.e. whether a susceptibility to be cheated exist in a clade
84 of partners; Merckx *et al.* 2012).

85

86 The arbuscular mycorrhizal symbiosis between plant roots and soil Glomeromycotina
87 fungi is a choice system for evaluating the strength of both types of constraints, as
88 cheating independently emerged multiple times. Indeed this mutualism is one of the
89 most ancient and widespread in terrestrial ecosystems, concerning c. 80% of extent land
90 plants and several hundreds fungal taxa (Davison et al. 2015). Arbuscular mycorrhizal
91 fungi provide host plants with water and mineral nutrients, in return for carbohydrates.

92 Although obligate, this symbiosis is generally unspecific and diffuse (van der Heijden et
93 al. 2015), since multiple fungi colonized most plants, and fungi are usually shared among
94 surrounding plant species (Verbruggen et al. 2012). Thus, fungi interconnect plant
95 individuals of different species, and move carbohydrates between plants (Selosse et al.
96 2006). This feature specifically allows the emergence of achlorophyllous cheating plants,
97 called mycoheterotroph (MH) plants, which obtain carbon from their mycorrhizal fungi
98 (Selosse & Rousset 2011). Unlike other cheating systems where cheating cost is shared
99 between partners (e.g. in plant pollination), MH cheaters involve not only a direct cost to
100 their fungal partners but also a projected cost (i.e. a cost transmitted through the
101 network) on the interconnected autotrophic (AT) plants, which supply their carbon.
102 Finally, we distinguish species that are entirely MH over their lifecycle (*EMH*), and
103 species that are only initially MH at early stage before turning AT at later stages (*IMH*).

104

105 Among preliminary insights about constraints upon MH cheating, it is believed that MH
106 plants are specialists interacting with few fungal species (Leake, 1994; Merckx, 2013).
107 However, whether or not these species are unusually specialized compared with overall
108 AT plants is still debated (Merckx *et al.* 2012). By specializing on few fungal species, MH
109 plants would evolve more efficient nutritional stealing strategies (Blüthgen et al. 2007)
110 and thus override interaction constraints. Mycorrhizal networks are generally nested
111 (Chagnon et al. 2012), but how MH plants affect the structure of mycorrhizal networks
112 has yet to be investigated. For instance, because they indirectly rely on AT plants for
113 carbon nutrition, we would expect MH species to interact more or less specifically with
114 highly generalist fungi, thereby having access to various carbon sources. Conversely,
115 because of the projected cost, AT plants could sanction interactions with fungi

associated with MH plants: this would isolate cheating interactions within antagonistic modules, although establishment of extreme reciprocal specialization seems also very unlikely in this system since a AT source is required. Otherwise, MH cheating strategies evolved multiple times (Merckx 2013), generating monophyletic families of exclusively MH plants, but Merckx et al. (2012) showed that fungi interacting with independent MH families are phylogenetically close. If this result were confirmed in a larger phylogenetic context including fungi of AT plants, it would evidence strong phylogenetic constraints upon MH emergence on the fungal side. Also, one interesting question has so far been disregarded: were these MH-susceptible fungi already present in the ancestral interactions of MH lineages, or were they horizontally acquired during MH emergence?

In this paper, we first develop a general framework in bipartite networks (interaction matrix coupled with available phylogenies) to assess the strengths of interaction and phylogenetic constraints upon cheaters emergence in mutualisms. We apply this framework to the largest database on arbuscular mycorrhizal fungi and their host plants (Öpik et al. 2013), which includes not only many AT plants but also the majority of MH plants. We investigate the phylogenetic distributions of MH plants and fungi in both phylogenies, and analyze the effects of MH interactions on the network specialization and structure.

Materials and Methods

Framework to assess the strength on constraints upon cheating

We developed an integrative framework to evaluate the strength of both interaction and phylogenetic constraints (Fig. 1). Firstly, interaction constraints expectedly affect the interactions between cheaters, cheated partners and other cooperative species. We used a global network approach, where all locally described interactions between species are merged and studied at larger scales (e.g. Joffard et al., 2018). By characterizing the relative specialization of cheaters and their partners and the overall structure of the global network (e.g. nestedness), we expect to snap the strength of interactions constraints on cheaters. We expect strong interaction constraints on the 'cheaters' side (resp. 'partners' side) to drive a specialization of cheaters toward few cheated partners (resp. of the cheated partners toward few cooperative species). Strong constraints on both sides drive reciprocal specialization and isolation of cheating into modules, and thus decrease nestedness.

Secondly, phylogenetic constraints expectedly affect the phylogenetic distributions of cheaters and their partners. We measured patterns of clustering and phylogenetically compared the sets of partners. We expect strong phylogenetic constraints on the 'cheaters' side (resp. 'partners' side) to drive a phylogenetic clustering of the cheaters (resp. of the cheated partners). Comparing the cheated partners to the partners of their closest cooperative relatives indicates whether sets of partners have drastically changed when cheaters emerged (i.e weak constraints), or derived from the inherited ancestral partners (i.e strong constraints).

160

161 The overall strength of constraints upon cheating (from weak effect to strong limitation)
162 may be deduced from an integrative analysis of these different patterns (Table S7).

163

164 ***MaarjAM database and interaction matrix***

165 MaarjAM is a web-based database (http://maarjam.botany.ut.ee; access in October
166 2017) of publicly available sequences of Glomeromycotina fungi, with information on
167 the host plants, geographical location and biomes for the recorded interactions (Öpik et
168 al. 2010, Davison et al. 2015). Among the 29,410 interactions between plants and
169 Glomeromycotina, we filtered out the data from MaarjAM to keep the fungi satisfying the
170 following criteria: (i) 18S rRNA gene sequence available, (ii) fungus identified from plant
171 roots (excluding soil samples and fungal cultures), (iii) interaction in a natural
172 ecosystem (excluding anthropogenic ecosystems), (iv) host plant identified at the
173 species level, and (v) a virtual taxon (VT) assignation is available in MaarjAM. In
174 MaarjAM, VT is a classification of arbuscular mycorrhizal fungi designed by applying a
175 ≥97% sequence similarity threshold to the 18S rRNA sequences, and by running
176 phylogenetic analysis to ensure VT monophyly (Öpik et al. 2013). The filtered dataset
177 yielded a worldwide binary interaction matrix of 344 plant species (hereafter called
178 'plant'), 314 VT (hereafter called 'fungus'), and 15,207 interactions.

179

180 ***Phylogenetic reconstructions***

181 We aligned consensus sequences of the 314 fungi with the software MUSCLE (Edgar
182 2004), and ran a Bayesian analysis using BEAST2 to reconstruct the fungal phylogeny
183 (Bouckaert *et al.* 2014, see Supplementary methods). The phylogenetic relationships

184 between the 344 host plants were obtained by pruning the time-calibrated supertree
185 from Zanne *et al.* (2013) using Phylomatic (http://phylodiversity.net/phylomatic/). We
186 also used the Open Tree of Life website (http://opentreeoflife.org) and the *rotl* package
187 (Michonneau et al. 2016) of R (R Core Team, 2018) for grafting 11 plant taxa missing in
188 the supertree (as polytomies at the lowest higher taxonomy level possible, see
189 Supplementary methods). Tree root calibrations were set at 505 million years (My) for
190 the fungi (Davison *et al.* 2015) and 440 My for the plants (Zanne *et al.* 2013).

191

192 ***Outcomes of the interactions***

193 We assigned each plant species to its outcome of its interaction with fungi according to
194 its carbon nutrition mode: autotroph (AT, n=310, 90.1%), entirely mycoheterotroph
195 (EMH, n=25, 7.3%), or initially mycoheterotroph (IMH, n=9, 2.6%). Identified on a on-
196 line database (http://mhp.myspecies.info/

197 category/myco-heterotrophic-plants/) and individual publications (Boullard, 1979;
198 Winther & Friedman, 2008; Field et al. 2015), EMH species of the dataset belong to five
199 monophyletic families [Burmanniaceae (13 spp.), Gentianaceae (6 spp.), Triuridaceae (4
200 spp.), Corsiaceae (1 sp.), and Petrosaviaceae (1 sp.)], and IMH species belong to the
201 families Ophioglossaceae (ferns; 3 spp.) and to the Lycopodiaceae (clubmoss; 6 spp.). We
202 assigned each fungus to three categories: 'AT-associated' if the fungus interacts with AT
203 plants <u>only</u> (n=260, 82.8%), 'EMH-associated' if the fungus interacts with <u>at least</u> one
204 EMH plant (n=40, 12.7%), 'IMH-associated' if the fungus interacts with <u>at least</u> one IMH
205 plant (n=15, 4.7%), or 'MH-associated' if the fungus interacts with <u>at least</u> one EMH or
206 IMH plant. Only *Glomus* VT130 was both EMH- and IMH-associated.

207

208

209 ***Network nestedness and specialization of cheaters***

210 In order to assess the strength of the interactions constraints, we tested the effect of MH
211 cheating on the network structure. Firstly, we measured nestedness in: (i) the global
212 network (344 plants, 314 fungi, and 15,207 interactions), (ii) the network of AT plants
213 (310, 308, and 15,016) and (iii) the network of EMH and IMH plants (34, 54, and 191),
214 using the function '*NODF2*' in the R package '*bipartite'* (Dormann et al. 2008). Positive
215 NODF values indicate nested networks.

216 We tested the significance of nestedness values by using two types of null models (*N*=
217 100 for each type): both maintain the connectance (proportion of observed interactions)
218 of the network, but the first model (*'r2dtable'* from *'stats'* R package - *null model 3*) also
219 maintains the marginal sums while the less stringent second model (*'vaznull'* from
220 '*bipartite'* R package - *null model 2*) produces slightly different marginal sums
221 (interactions are randomized with species marginal sums as weights, and each species
222 must have at least one interaction). We calculated the Z-score, which is the standard
223 deviation between the observed value and the mean of the distribution of null-model
224 networks (Z-scores greater than 1.96 validates a significant nestedness with a alpha risk
225 of 5%).

226 Secondly, the detection cases of extreme reciprocal specializations, leading to
227 independent modules, was performed using projected degrees, i.e. degrees obtained by
228 creating a unipartite network of plants interacting together if they share at least one
229 fungus (the degree (*k*) is the number of partners with which a given plant or fungus
230 interacts in the network).

231 Thirdly, to further evaluate the specialization of MH cheaters, several network indices
232 we calculated for each plant and fungus (i.e., each node) in the network. The degree (*k*)
233 is high (resp. low) when the species is generalist (resp. specialist). The partner
234 specialization (*Psp*) is the mean degree (*k*) averaged for all the partners for a given plant
235 or fungus (Taudiere *et al.* 2015): a high (resp. low) *Psp* characterizes a species
236 interacting mainly with generalist (resp. specialist) partners. Low degree *k* and partner
237 specialization *Psp* are signs of reciprocal specialization.

238 In order to detect specialization at clade scale toward partners, for any given clade of
239 every node in the plant or fungus phylogenies, we calculated the partner fidelity *Fx* as
240 the ratio of partners exclusively interacting with this particular clade over the total
241 number of partners interacting with it. We consider the clade as 'faithful' and the
242 corresponding set of partners as 'clade-specific' when *Fx*>0.5 (i.e., more than 50%
243 exclusive partners).

244 We tested whether the network indices *k* and *Psp* were statistically different among AT,
245 EMH and IMH plants using non-parametric Kruskal-Wallis tests and pairwise Whitney U
246 tests. We also replicated the statistical tests without the IMH Lycopodiaceae, which
247 showed different network patterns (see Results). We use an analysis of covariance
248 (ANCOVA) to test the effect of the interaction outcomes on partner fidelity *Fx* accounting
249 for clade size, which corrects the bias of having high partner fidelity *Fx* in older clades
250 including many plant species. Similarly, analyses were conducted from the fungal
251 perspective to test the effect of the three categories 'AT-associated', 'EMH-associated'
252 and 'IMH-associated'.

253 To assess the significance of *k* and *Psp* values, null-model networks (*N*= 100) were built
254 using the function '*permatfull'* in '*vegan'* R package *(null model 1)* with same connectance
255 but different marginal sums.

256

257 ***Phylogenetic distribution of cheating***

258 In order to assess the strength of the phylogenetic constraints, we explored the
259 phylogenetic distribution of MH cheaters and MH-associated fungi. Firstly, we
260 investigated the phylogenetic distribution of mycoheterotrophy, i.e. if MH plants and
261 MH-associated fungi are more or less phylogenetically related than expected by chance
262 (i.e. patterns of clustering versus overdispersion). We computed the net relatedness
263 index (NRI) and the nearest taxon index (NTI) using the '*PICANTE'* R package (Kembel et
264 al. 2010). NRI quantifies the phylogenetic structure of a species set based on the mean
265 pairwise distances, whereas NTI quantifies the terminal structure of the species set by
266 computing the mean phylogenetic distance to the nearest taxon of every species (Gotelli
267 & Rohde 2002). To standardize the indices, 999 null models were generated for each of
268 the following option '*taxa.labels'* (shuffles the taxa labels) and '*phylogeny.pool'*
269 (randomizes interactions by affecting probability to species according to phylogenetic
270 distances). Significant positive (resp. negative) NRI and NTI values indicate phylogenetic
271 clustering (resp. overdispersion). We computed these indices (i) on the plant phylogeny
272 to evaluate the phylogenetic structure of EMH and IMH plants distribution, and (ii) on
273 the fungal phylogeny to investigate if MH-associated fungi are phylogenetic structured
274 (we tested the distribution of MH-, EMH-, and IMH-associated fungi, and then of the
275 fungi associated with each specific MH family). Similarly, for each species, we computed
276 the partners' mean phylogenetic pairwise distance (*MPD*) that is the mean of

277 phylogenetic distances between every pair of partners (Kembel *et al.* 2010): a low value
278 of *MPD* indicates that the set of partners is constituted of closely related species. The
279 effect of the MH cheating on *MPD* values and its significance was evaluated as for *k* and
280 *Psp* values.

281 Secondly, we aimed to compare in an evolutionary framework the sets of fungi
282 associated with plants with different interaction outcomes. To do so, we computed the
283 unweighted Unifrac distance (Lozupone & Knight 2005) between sets of fungi
284 interacting with each pair of plants in the network. For each one of the seven MH
285 families, we compared the Unifrac distances across (i) every pairs of plant species of this
286 family, (ii) every pairs made of one plant of this family and one plant of the most closely
287 related AT family (see Table 2 for AT families), (iii) every pairs made of one plant of this
288 family and one plant belonging to other MH families, and (iv) every pairs made of one
289 plant of this family and one more distant AT plant (i.e. all AT plants except those of the
290 most closely related AT family). Differences between groups of distances were tested
291 using Mann-Whitney U tests.
292

293 **Results**

294 We analysed the global arbuscular mycorrhizal interaction network filtered from the
295 MaarjAM database combined with the reconstructed phylogenetic trees of both partners
296 (Fig. 2).

297

298 ***Network nestedness and specialization of cheaters***

299 The global network had a significant positive nestedness value (Z-score=11.0, P=1.10^{-28},
300 Table S3). Nestedness increased when considering only AT plants (Z-score=14.3, P=6.10^{-}
301 ^{47}), whereas nestedness was not significant in the network of only MH plants (Z-
302 score=0.79, P=0.21): MH plants weakened nestedness, signifying higher reciprocal
303 specializations in MH interactions. On the side of a main module encompassing most
304 species (335 out of 344 plants and 309 out of 314 fungus; 99,8% interactions), three
305 small independent modules were found (Table S2), as follows: all 6 IMH Lycopodiaceae
306 plants and their 3 fungi (*Glomus* VT127, VT158, VT394); two AT plants from salt
307 marshes (*Salicornia europaea* and *Limonium vulgare*) with one *Glomus* (VT296); and the
308 EMH *Kupea martinetugei* with a unique *Glomus* (VT204).

309 From the observed degrees (*k*), EMH and IMH plants are significantly more specialized
310 than AT plants by interacting with less fungi (P=$2.8.10^{-13}$, Kruskal-Wallis test; Fig. 3A,
311 Table 1). On the fungal side, EMH- or IMH-associated fungi interact with a lower number
312 of plants than AT-associated fungi (P=0.04). Indeed, partner specializations (*Psp*)
313 confirm that MH plants interact with more specialized fungi (i.e. MH-associated fungi
314 interact on average with fewer plants; P=$7.3.10^{-9}$; Fig. 3A). Reciprocally, on the fungal
315 side, plants interacting with EMH- and IMH-associated fungi are more specialized than
316 plants interacting with AT-associated fungi only (P=0.0005; Table 1). We found similar

317 evidence for MH reciprocal specializations by reanalyzing the network excluding the
318 family Lycopodiaceae (Table 1). Significance assessments using null models are in
319 supplementary files (Table S4).

320 According to the partner fidelity (*Fx*), very few plant and fungi clades interact with
321 'clade-specific' partners (i.e. *Fx*>0.5). Instead, most fungi are shared between different
322 plant clades (Fig. 3C). Among exceptions, however, the whole family Lycopodiaceae
323 presents a high partner fidelity (*Fx*=1), encompassing the entire Lycopodiaceae-
324 associated fungi clade (Fig. S3). Similarly, the ANCOVA testing the effect of interactions
325 outcomes on partner fidelity (accounting for clade size) confirmed that Lycopodiaceae is
326 the only plant clade significantly presenting a high partner fidelity ($P=3.5.10^{-66}$; Fig. 3C).

327 While the Lycopodiaceae and their fungal partners were previously shown to form an
328 independent module, this result demonstrates that the Lycopodiaceae-associated fungi
329 form actually a monophyletic clade within Glomeromycotina. The estimated age was 265
330 My for the clade Lycopodiaceae and 44 My for the clade encompassing Lycopodiaceae-
331 associated fungi (Fig. 3D), which diverged 69 My ago from the other *Glomus* fungi.

332

333 ***Phylogenetic distribution of cheating***

334 Regarding the partners' mean phylogenetic pairwise distance (*MPD*), both EMH- and
335 IMH-associated fungi are phylogenetically more closely related than AT-associated fungi
336 ($P=1.6.10^{-6}$; Table 1; Fig. 3B), and reciprocally, they also interact with more closely
337 related plants ($P=1.2.10^{-4}$). NRI and NTI values (Table S6) confirm a significant
338 clustering of MH-associated fungi, EMH-associated and IMH-associated fungi on the
339 fungal phylogeny; this clustering hold at the family level for four main MH families
340 (namely Burmanniaceae, Triuridaceae, Lycopodiaceae and Ophioglossaceae). For the

341 plants, only the EMH plants are significantly clustered, and not all MH plants in general,
342 nor the IMH plants (Table S6). This phylogenetic clustering is visually noticeable on
343 fungal and plant phylogenetic trees (Fig. S1 and S2). This shows that MH cheating has
344 evolved several times independently in plants, but interact mainly with closely related
345 fungi (see also Fig. 2).

346 Looking specifically at the shared fungi highlighted differences between EMH and IMH
347 plants (Table 2). While the IMH Lycopodiaceae family forms an independent module
348 with three private *Glomus*, the other IMH family Ophioglossaceae also interacts with 2
349 fungi exclusive to this fern family over a total of 12 fungi (*Glomus* VT134 and VT173).
350 When comparing the fungi shared between MH families (Table 2), mainly two closely
351 related families, namely Burmanniaceae and Triuridaceae, tend to share some fungi with
352 other MH families.

353 By comparing the Unifrac dissimilarities between set of fungal partners according to the
354 interactions outcome and plant family relatedness, we observed two tendencies among
355 MH families (Fig. 4; Table S5). Two EMH families, Burmanniaceae and Triuridaceae, had
356 fungal partners significantly more similar to partners interacting with their closest AT
357 relatives ($P>0.05$) than to partners interacting with other AT families ($P<4e-03$).
358 Additionally, the fungal partners of families Burmanniaceae and Triuridaceae were also
359 not significantly different from fungal partners interacting with other MH families. For
360 other families of EMH (Corsiaceae, Gentianaceae and Petrosaviaceae) or IMH
361 (Lycopodiaceae and Ophioglossaceae), fungal partners were significantly more similar
362 to partners interacting with other MH families than to partners interacting with their
363 closest AT relatives (Table S5).

364 **Discussion**

365

366 We proposed a framework to assess the strengths of constraints upon mycoheterotroph
367 (MH) cheating emergences in the mutualistic networks linking plants and arbuscular
368 mycorrhizal fungi. Firstly, we found a trend toward reciprocal specializations: specialist
369 MH cheaters seem constrained to interact with rather specialist fungi. We even observed
370 unexpected, extreme reciprocal specializations for IMH families interacting with
371 'private' fungi. Secondly, the phylogenetic comparisons of fungal partners highlighted
372 strong similarities between set of fungi interacting with independently emerged MH
373 families, which either derived from AT mutualistic relatives, or have been horizontally
374 acquired. We discuss these results based on the current understanding of the arbuscular
375 mycorrhizal symbiosis, and argue the roles of constraints upon cheaters in stabilizing
376 mutualisms.

377

378 ***Cheaters are isolated by reciprocal specialization***

379 Cheaters lower the nestedness in the mycorrhizal network: we confirmed that MH
380 plants are more specialized on fewer fungal partners (Merckx et al., 2012), and showed
381 that their fungal partners reciprocally are more specialist than fungi exclusively
382 associated with AT plants. This contrasts with our prediction from network theory of
383 nested mutualisms that specialist cheaters interacting with generalist partners (i.e.
384 asymmetrical specialization) would be more favored and resilient (see Introduction):
385 the observed trend to reciprocal specialization looks an unstable ecological and
386 evolutionary strategy for cheating. This instability could explain the relative youngness
387 of MH clades (Fig. 2), because these cheating clades would not persist on long
388 evolutionary timescale.

389

390 Several alternative, non-exclusive reasons may explain this reciprocal MH specialization.
391 Firstly, this pattern may very likely result from strong interaction constraints.
392 Conditional investment and partner choice occur between plants and their fungal
393 partners (Kiers et al. 2011): plants and fungi reward and interact specifically with the
394 most mutualistic of the many partners they encounter in soil. On the plant side, MH
395 cheaters may have avoided this constraint in few specific MH-susceptible fungi (Selosse
396 & Rousset, 2011) with which they are now faithfully interacting through a parasitic
397 specialization. On the fungal side, fungi transferring nutrients to MH plants have greater
398 carbon costs for AT plants than other competing cooperative fungi. Consequently, AT
399 plants' partner choices, tend to minimize interactions with these costlier MH-associated
400 fungi, which themselves will specialize on few susceptible AT plants. Reciprocal
401 specialization would result from these interaction mechanisms, and lead to an isolation
402 of MH cheating in the mutualistic network.

403

404 Secondly, MH cheaters might also be limited by the partners they can parasitically
405 interact with: the MH-associated fungi are maybe less cooperative partners by
406 themselves, and therefore intrinsically rejected by autotrophs. Cheating plants could
407 then mainly be interacting with 'cheating' fungi.

408

409 Thirdly, we analysed here a global network built from mycorrhizal interactions
410 described in different ecosystems around the world. Such global analysis is justified by
411 the very low endemism of arbuscular mycorrhizal fungi, which guarantees the absence
412 of strong geographic structure (Davison et al. 2015). However, a species can be very
413 generalist in the local community where actual interactions develop, while it appears

414 nevertheless as intermediately generalist at the global scale. Thus, while MH-associated
415 fungi are specialist in the global network, they might be relatively generalist within the
416 local communities where MH plants live. Moreover, our approach did not consider
417 species abundances. A plant species can be specialist at the global scale, but locally
418 largely abundant, and thus being an advantageous target for MH plants, despite
419 apparent reciprocal specialization. All these potential biases may only be dismissed
420 thanks to an in-depth sampling of the abundances of mycorrhizal interactions in a MH
421 local community.
422
423 Whatever its origin, the specialization of cheaters was also suggested in other
424 mutualisms (Genini et al. 2010). A parasitic nature of EMH plants has often been guessed
425 without direct support (Bidartondo, 2005; Merckx, 2013) in the absence of data on
426 fitness of fungal partners and surrounding AT plants (van der Heijden *et al.* 2015). Our
427 analysis is *a posteriori* congruent with the view of EMH plants as parasitic cheaters.
428
429
430 ***MH cheating emergences convergently target similar susceptible fungi***
431 Our approach confirms that fungal clades interacting with independently emerged MH
432 cheaters are not numerous and phylogenetically related (Merckx et al., 2012).
433 Although MH-associated fungi turn out to be very similar and phylogenetically
434 constrained, the multiple emergences of cheating at different position of the plant
435 phylogeny indicate that the overall strength of phylogenetic constraints is low.
436 This weakness likely results from the low specificity in the arbuscular mycorrhizal
437 symbiosis: convergent interactions (Bittleston *et al.* 2016) arose in different plant clades
438 toward similar MH-susceptible fungi. Thus, interaction constraints, shaping MH isolation

439 by reciprocal specialization, is likely the only barrier to cheating emergences in the
440 arbuscular mycorrhizal mutualism.
441
442 The susceptibility of fungi is difficult to discuss, because we know little about
443 physiological and symbiotic traits of these diverse microscopic fungi (Chagnon et al.
444 2013; van der Heijden & Scheublin 2007). Yet, interactions between MH cheaters and
445 MH-susceptible fungi likely represent exceptions to the partner choice, which is
446 generally considered to constitutively maintain the stability of this mutualism (Selosse &
447 Rousset 2011).
448
449 Our comparisons of the sets of fungal partners according to interactions outcome and
450 plant family relatedness show two evolutionary pathways. Firstly, the similarity
451 between fungi associated with the closely related MH families Burmanniaceae and
452 Triuridaceae (order Dioscoreales) and fungi associated with their most closely related
453 AT family highlights a phylogenetic conservatism of partners: MH-associated fungi
454 would have been inherited and derived from the fungal partners of cooperative AT
455 ancestors. Secondly, the MH families Corsiaceae, Gentianaceae, and Petrosaviaceae
456 interact with fungi more similar to fungi interacting with other MH families than to fungi
457 interacting with their closest AT relatives: MH-associated fungi would not derived from
458 the fungal partners of cooperative AT ancestors, but would have been secondly acquired
459 from the suitable set of MH-susceptible fungal partners other MH families.
460
461 The Dioscoreales (including Burmanniaceae and Triuridaceae) is the oldest clade
462 including EMH plants (approximately 130 My; Zanne et al. 2013; Fig. 2). Thus, we
463 speculate that the mycoheterotrophy we observe in EMH plants initially emerged in the

464 Dioscoreales clade, and secondly, thanks to the weak phylogenetic constraints, the more
465 recently evolved EMH plant families convergently reutilized the cheating-susceptible
466 fungal partners.
467
468 ***Private networks and parental nurture in IMH***
469 Among all MH families, only IMH Ophioglossaceae and Lycopodiaceae surprisingly
470 interact with exclusive fungi, which do not interact with any other plant families. These
471 fungi are present during MH underground germination and in roots of adult AT
472 individuals (Winther & Friedman, 2007, 2008). Thus, their carbon source is likely from
473 AT adult. Leake *et al.* (2008) hypothesized of parental nurture where germinating
474 spores would be indirectly nourished by surrounding adults: our data further support
475 cases of parental nurture mediated by exclusive fungi forming private networks.
476 Importantly, such interactions may not be cheating, since their overall outcome over the
477 plant lifespan is positive, because fungi invest in MH germinations that represent future
478 carbon sources ('take now, pay later' model; Field et al. 2015). In other words, our
479 analysis reveals how IMH plants do not cheat their exclusive fungi, but postpone reward.
480
481 Particularly, we demonstrated the extreme reciprocal specialization between
482 Lycopodiaceae and their exclusive clade of *Glomus*. From a different dataset, Rimington
483 *et al.* (2018) showed that early-diverging plant clades interact with early-diverging
484 fungal clades with the exception of Lycopodiaceae, independently confirming our
485 pattern. Lycopodiaceae is a 265-My-old clade whereas its fungal partners form a 44-My-
486 old clade of 3 *Glomus* diverged 69 My ago from all other *Glomus*. Thus, this highly
487 specific interaction would be a secondly evolved acquisition in Lycopodiaceae.
488 Lycopodiaceae may have transiently developed IMH cheating from a more generalist

489 state, which later evolved into a specific mutualistic cooperation by way of parental
490 nurture, suggesting a strong linked evolution between both clades.
491
492 Thus, our framework challenges the cheater status of mycoheterotrophy by highlighting
493 the different evolutionary meaning of EMH and IMH cheaters: it reveals a dichotomy
494 between true MH cheating and private MH systems likely cooperative. It further
495 supports the interest of interaction network analyses coupled with phylogenetic
496 analyses in unraveling and discussing cheating *versus* cooperative processes.
497

498 **Conclusions**

499 Our analysis of mycoheterotroph cheating in the arbuscular mycorrhizal symbiosis
500 shows how strong interaction constraints prevent the emergence of cheaters in
501 mutualisms. Cheating emergence seems to be compromised by reciprocal specialization:
502 partner choices or sanctions break the nested network into modules, isolating cheaters
503 and their 'cheating-susceptible' partners. Conversely, we detected limited phylogenetic
504 constraints: independently emerged cheaters interact with very similar closely related
505 partners, implying that cheaters convergently interacted with cheating-susceptible
506 partners.
507 Although well-established in several independent clades, cheating is constrained to
508 remain rather infrequent and marginally challenge the stability of the mutualism.
509 Beyond the mycorrhizal symbiosis, we invite to use our framework to evaluate the
510 strength of constraints upon cheating in other multiple-partner mutualisms (e.g.
511 pollination or seed dispersal).
512
513

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633

Tables & Figures

Constraints upon cheating emergence in mutualistic networks: evidence in the mycorrhizal symbiosis

Tables (1 – 2)

Table 1: Effect of interaction outcomes (i.e. plant carbon nutrition modes) on indices of network structure and phylogenetic distributions:
In black: P-values of Kruskal-Wallis tests (each index as a function of the interaction outcome) for the global network. P-values in brackets were calculated in the network without the Lycopodiaceae.
Bold values highlight the indices significantly different according to the interaction outcome (threshold of 5%).
In grey: P-values of Whitney U tests (pairwise tests for each index according to two interaction outcomes) for the global network including Lycopodiaceae.

Index	Plants			Fungal partners		
	AT vs. EMH	AT vs. IMH	IMH vs. EMH	AT vs. EMH	AT vs. MH	IMH vs. EMH
Degree (*k*)	**2.8e-13 (5.8e-11)**			**0.050 (0.039)**		
	2.4e-11	**8.5e-05**	0.60	**0.013**	0.92	0.34
Partner specialization (*Psp*)	**7.3e-9 (3.8e-6)**			**4.7e-4 (0.011)**		
	7.1e-7	**1.7e-5**	**9.9e-3**	0.28	**1.3e-5**	**4.5e-3**
Partner phylogenetic mean pairwise distance (*MPD*)	**1.6e-6 (1.1e-4)**			**1.2e-4 (6.1e-4)**		
	8.6e-4	**6.2e-5**	**7.3e-3**	**2.6e-3**	**1.4e-3**	0.062
Partner fidelity (*Fx*)	**3.5e-66 (2.6e-3)**			**6.4e-12** (/)		
	0.036	**9.0e-4**	**4.0e-3**		**0.017**	

656

657

658

659 **Table 2:** Fungal sharing between mycoheterotroph (MH) cheating families:
660 Number of shared fungi (matrix lower part) and percentage of shared fungi (matrix
661 upper part) between pairs of MH families. The two last columns represent (i) the total
662 number of fungi shared with other MH families, and (ii) the number of fungi specific to
663 this family. The second column indicate, for each MH family, its most closely related AT
664 sister-clade (can be either one family, or a clade of families, or none in the case of the
665 early-diverging isolated family Lycopodiaceae).

666

		Most closely related AT sister-clade in our dataset	**Number of plant species**	**Number of fungal partners**	Burmanniaceae	Corsiaceae	Gentianaceae	Petrosaviaceae	Triuridaceae	Lycopodiaceae	Ophioglossaceae	**Total number of shared fungi**	**Total number of specific fungi**
EMH	**Burmanniaceae**	Dioscoreaceae	13	29		0%	13%	3%	14%	0%	3%	10	0
	Corsiaceae	Melanthiaceae Liliaceae	1	1	0		0%	0%	0%	0%	0%	0	0
	Gentianaceae	Apocynaceae	6	7	4	0		0%	6%	0%	0%	4	0
	Petrosaviaceae	Dioscoreaceae Melanthiaceae	1	2	1	0	0		0%	0%	0%	1	0
	Triuridaceae	Dioscoreaceae	4	11	5	0	1	0		0%	0%	5	1
IMH	**Lycopodiaceae**		6	3	0	0	0	0	0		0%	0	3
	Ophioglossaceae	Gleicheniaceae Lygodiaceae Osmundaceae Tectaridaceae	3	12	1	0	0	0	0	0		1	2

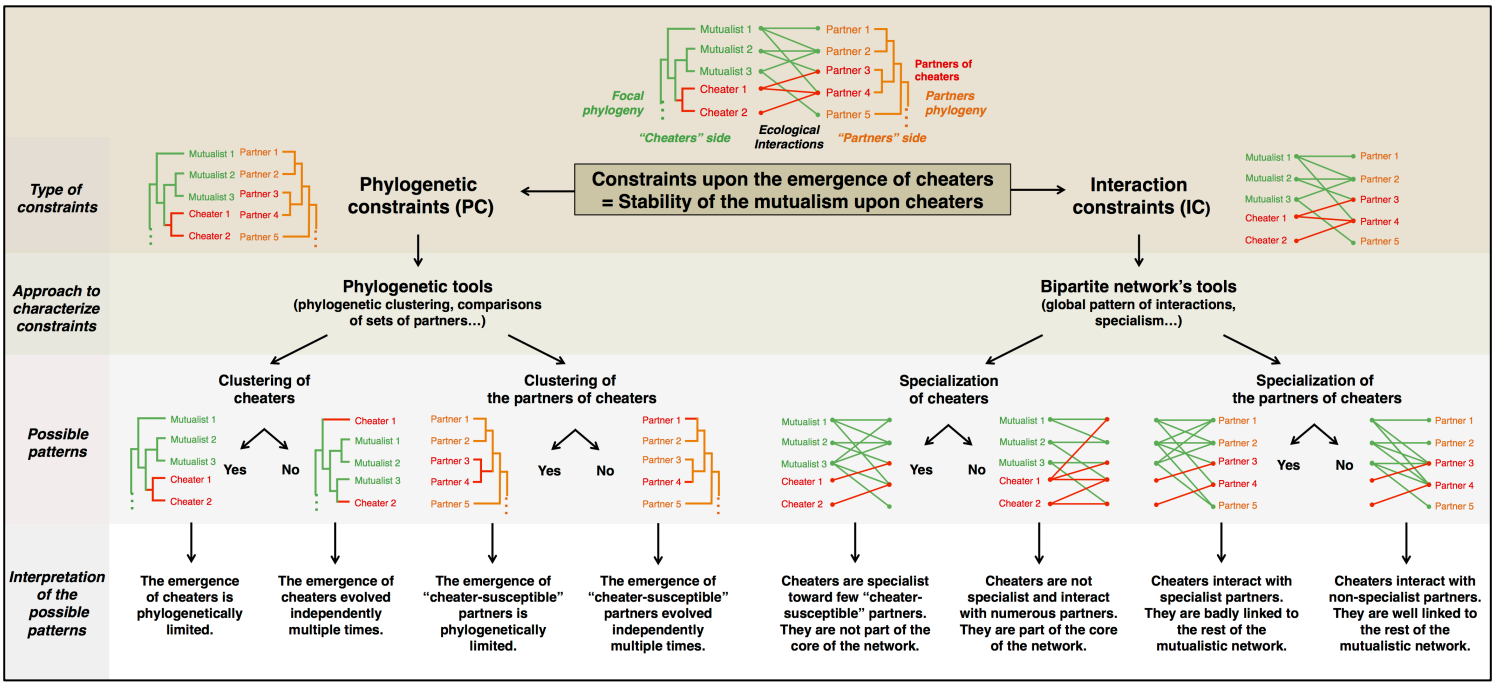
667

668

Figures (1 – 4)

Figure 1: Framework to evaluate the strengths of constraints upon cheating emergences in multiple-partner mutualisms.

This general framework proposes to evaluate in parallel the interaction constraints (IC) phylogenetic constraints (PC) upon cheating emergences. These constraints affect the structure of the interaction network (level of specialization of cheaters and their partners) and the phylogenetic distributions of cheating (phylogenetic clustering of the cheaters and their partners). Therefore, our approach is based on the investigation of specializations and phylogenetic clusterings on the "cheater side" and on the "partner side". These investigated patterns are considered as proxies of the strengths of both constraints on both sides. Possible combinations of the strengths of phylogenetic constraints and interaction constraints and their overall consequences on the stability of the mutualism are presented in Table S7.



686 **Figure 2:** Mycoheterotroph (MH) cheating in the global arbuscular mycorrhizal
687 mutualism:
688 Plant phylogenetic tree (left - 344 plant species) and fungal tree (right - 314 arbuscular
689 mycorrhizal fungi) are linked by the observed interactions between plants and fungi
690 (15,207 interactions). The colors of the links correspond to the interaction outcome (i.e.
691 the plant carbon nutrition modes, which are AT: autotrophic; EMH: entirely
692 mycoheterotrophic; and IMH: initially mycoheterotrophic). Main plant and fungal clades
693 are captioned. Cheaters encompass 25 EMH plants in 5 families, and 9 IMH in 2 families.

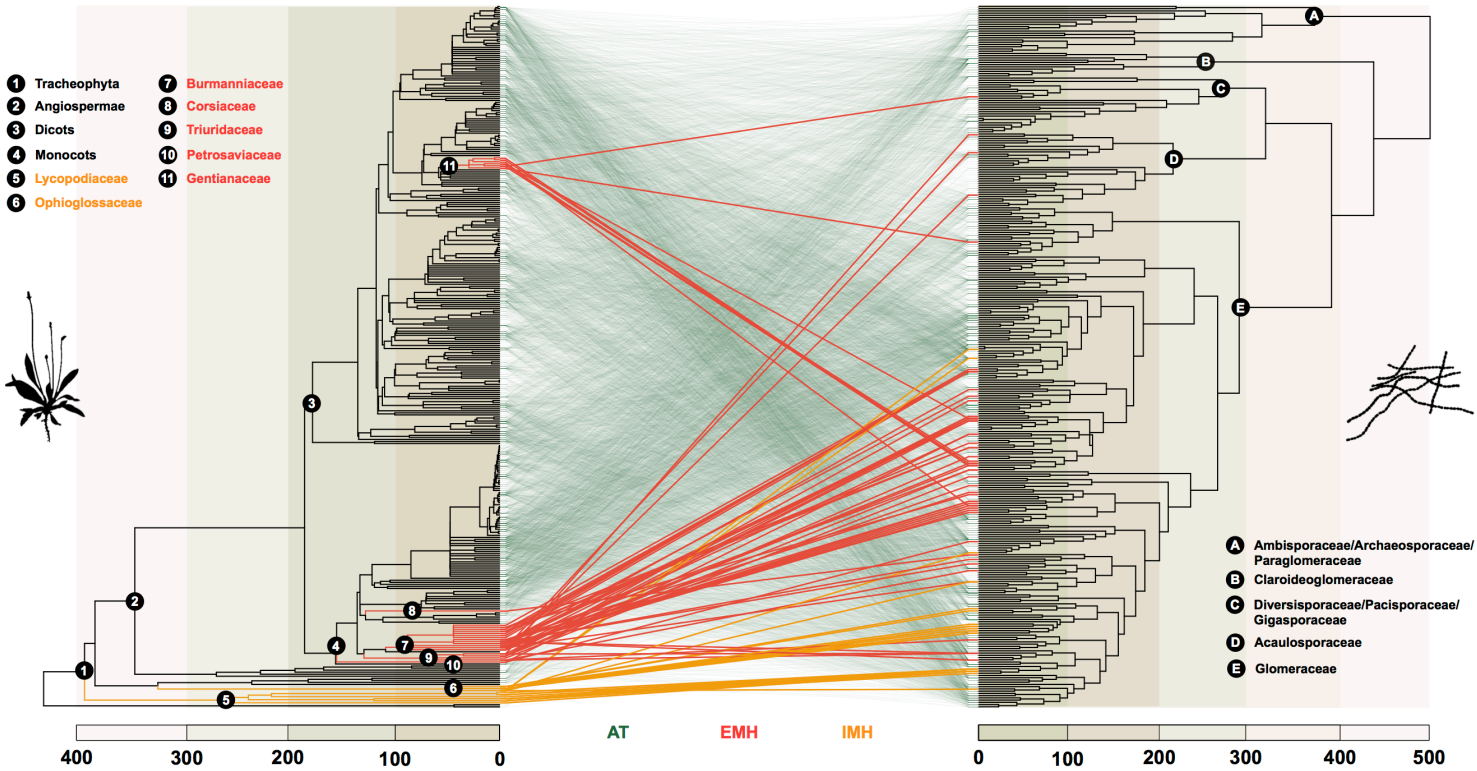


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Figure 3: Effect of the interaction outcomes on specializations (*k* and *Psp*), partner's phylogenetic distributions (*MPD*), and partner fidelity (*Fx*):
(Categories are defined according to the plant carbon nutrition modes, i.e. AT: autotrophic; EMH: entirely mycoheterotrophic over development; and IMH: initially mycoheterotrophic in the development).
A: plant degree (*k*) against plant partner specialization (*Psp*) (i.e. average degree of the arbuscular mycorrhizal fungal partners); dots in the bottom left corner indicate reciprocal specialization.
B: Partner's mean phylogenetic pairwise distance (*MPD*) of the sets of fungal partners. Boxplots present the median surrounded by the first and third quartile, and whiskers extended to the extreme values but no further than 1.5 of the inter-quartile range.
C: Fidelity toward fungal partners in relation to the age of the plant clade; note that brown dots (clades of Lycopodiaceae) depart from other MH clades. Clades are defined according to their main carbon nutrition mode of their plants (over 50%).
D: Independent interaction sub-network between of the clubmoss family Lycopodiaceae (rows) and their three arbuscular mycorrhizal fungi (columns) with their respective phylogenetic relationships.

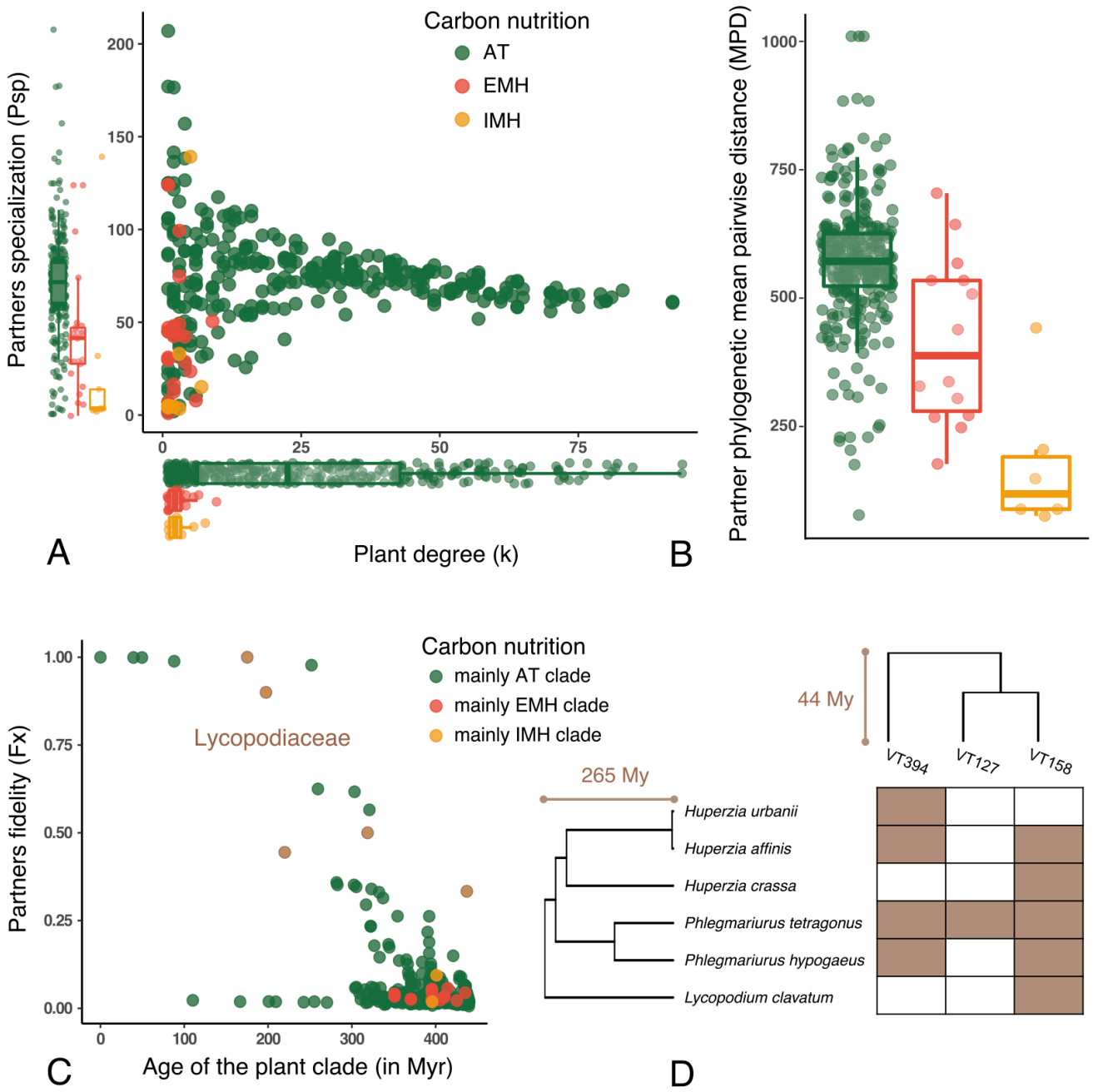
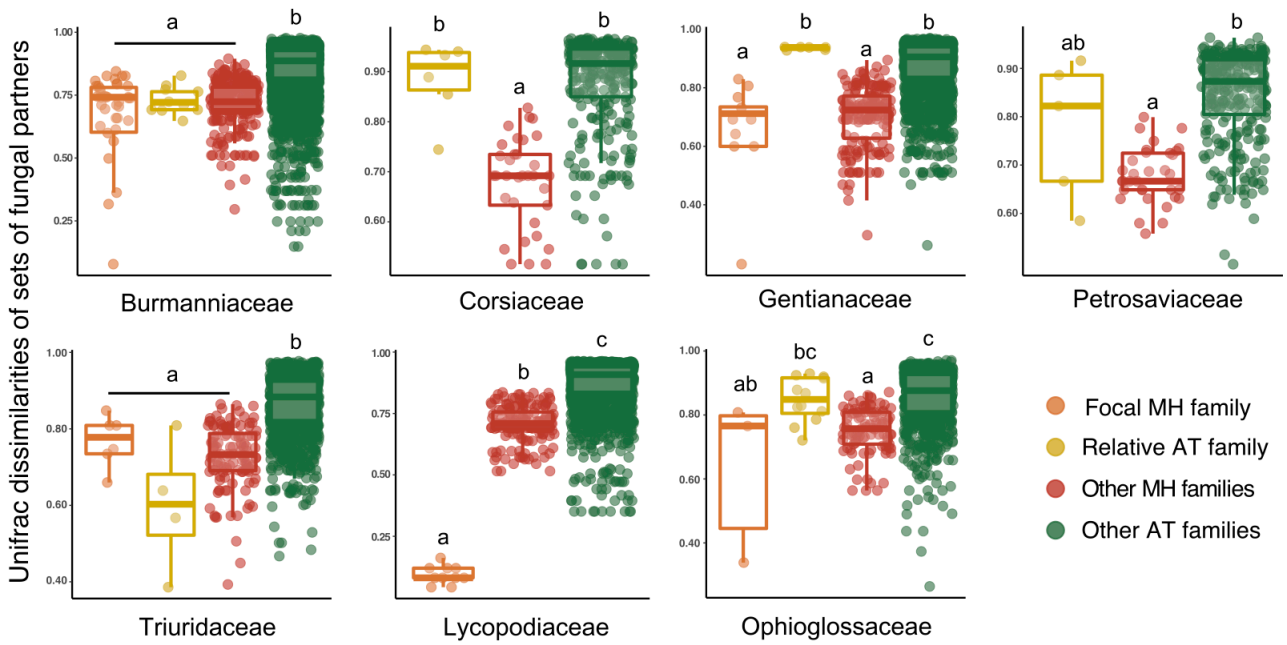

Carbon nutrition
AT
EMH
IMH
Partners specialization (Psp)
Plant degree (k)
A
Partner phylogenetic mean pairwise distance (MPD)
B
Carbon nutrition
mainly AT clade
mainly EMH clade
mainly IMH clade
Lycopodiaceae
Partners fidelity (Fx)
Age of the plant clade (in Myr)
C
265 My
44 My
VT394
VT127
VT158
Huperzia urbanii
Huperzia affinis
Huperzia crassa
Phlegmariurus tetragonus
Phlegmariurus hypogaeus
Lycopodium clavatum
D

Figure 4: Dissimilarities between sets of fungal partners associated with different MH plant families:

For each MH family, Unifrac dissimilarities of sets of fungal partners are calculated between one particular MH species belonging to the focal MH plant family and another plant species (from the same family, from the closest related AT family, from other MH families, or from other AT plant families). All the groups cannot be calculated for every MH family, due to: (i) single species within families Corsiaceae and Petrosaviaceae, and (ii) difficulty to define the most closely related AT family for Lycopodiaceae.